

Identification of Equine Cecal Bacteria Producing Amines in an In Vitro Model of Carbohydrate Overload
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Acute laminitis has been associated with the overgrowth of gram-positive bacteria within the equine hindgut, causing the release of factor(s) leading to ischemia-reperfusion of the digits. The products of fermentation which trigger acute laminitis are, as yet, unknown; however, vasoactive amines are possible candidates. The objectives of this study were to use an in vitro model of carbohydrate overload to study the change in populations of cecal streptococci and lactobacilli and to establish whether certain species of these bacteria were capable of producing vasoactive amines from amino acids. Cecal contents from 10 horses were divided into aliquots and incubated anaerobically with either corn starch or inulin (fructan; both at 1 g/100 ml). Samples were taken at 6-h intervals over a 24-h period for enumeration of streptococci, lactobacilli, and gram-negative anaerobes by a dilution method onto standard selective growth media. The effects of the antibiotic virginiamycin (1 mg/100 ml) and calcium hydrogen phosphate (CaHPO₄; 0.3 g/100 ml) were also examined. Fermentation of excess carbohydrate was associated with increases in numbers of streptococci and lactobacilli (2- to 3.5-log unit increases; inhibited by virginiamycin) but numbers of gram-negative anaerobes were not significantly affected. A screening agar technique followed by 16S rRNA gene sequence analysis enabled the identification of 26 different bacterial strains capable of producing one or more vasoactive amines. These included members of the species *Streptococcus bovis* and five different *Lactobacillus* spp. These data suggest that certain bacteria, whose overgrowth is associated with carbohydrate fermentation, are capable of producing vasoactive amines which may play a role in the pathogenesis of acute laminitis.

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Fermentation of carbohydrate in the hindgut has been recognized as one of the primary events associated with acute laminitis in the horse (14). The ability of the equine small intestine to digest starch may be overwhelmed if starch is present in excess (>0.4% body



weight [35]), leading to its metabolism by cecal and colonic bacteria and the production of lactic acid. Fructans, the oligosaccharides present in grass which act as the storage form of carbohydrate, do not appear to be digested by the small intestine (21) and so are also substrates for fermentative bacteria. The variation of fructan levels in grasses under different climatic conditions has been suggested to account for the seasonal incidence of acute laminitis (A. C. Longland, A. J. Cairns, and M. O. Humphreys, Proc. 16th Equine Nutr. Physiol. Soc., p. 258-259, 1999).

Gram-positive bacteria have been implicated in the pathogenesis of laminitis in the carbohydrate overload model since they have been shown to overgrow in the cecum of horses given corn starch by stomach tube (16). This overgrowth, particularly of streptococci and lactobacilli, was associated with lactic acid accumulation and the death of gram-negative bacteria leading to endotoxin release, both of which were postulated to be the cause of this condition (15, 31). Although endotoxin causes widespread inflammatory effects in the horse, on its own it does not induce laminitis (D. M. Hood, Proc. Am. Assoc. Equine Pract., vol. 41, p. 245-247, 1995). Another theory, put forward by Mungall et al. (33), suggests that exotoxins produced by *Streptococcus bovis* may induce enzymatic degradation of the lamellar basement membrane. However, the primary factor(s) which trigger the clinical syndrome of acute laminitis remain to be fully elucidated.

Amines, vasoactive compounds produced by the decarboxylation of amino acids by bacteria (24, 37), may also be capable of triggering changes seen in the developmental phase of acute laminitis. These compounds may be produced by gram-positive bacteria including streptococci and lactobacilli (18, 28) as well as gram-negative species and are present in high concentrations in the normal equine cecum (5). Furthermore, concentrations of a number of amines were found to be significantly increased when excess amounts of starch or fructans were added to cecal contents incubated in vitro (5). The effects of these compounds, should they gain access to the peripheral circulation at sufficient concentrations, may include digital vasoconstriction, due to direct and indirect interactions with serotonin (5-hydroxytryptamine) and adrenoreceptors (S. R. Bailey, Y. Berhane, C. M. Marr, and J. Elliott, Abstract, J. Vet. Intern. Med. 15:374, 2001; Y. Berhane, S. B. Bailey, and J. Elliott, Abstract, Br. J. Pharmacol. 135:291P, 2002). Serotonin is a particularly potent vasoconstrictor of equine digital arteries and veins (4, 6). Although not universally accepted, digital lamellar ischemia followed by reperfusion injury is thought to be the sequence of events leading to acute equine laminitis (22, 23).

Virginiamycin, a streptogramin antibiotic, has been used to prevent starch overload-induced laminitis, by preventing the overgrowth of gram-positive bacteria which ferment carbohydrate and produce lactic acid (40). It is not known whether it is the inhibition of lactate production per se and/or inhibition of the formation and absorption of other bacterial products which mediates this protective effect. Furthermore, dietary calcium in the form of CaHPO₄ has been shown to modulate inulin-induced cecal fermentation in the rat, partly by control of luminal pH (36), but has not been investigated in other species in this regard.

The present study describes the changes in populations of streptococci, lactobacilli, and gram-negative anaerobes in equine cecal contents, using an in vitro model of

carbohydrate overload. The effects of virginiamycin and CaHPO_4 on these bacterial populations were also studied. The changes in pH and concentrations of vasoactive amines recorded in these experiments have been reported elsewhere (5). Isolates of streptococci and lactobacilli obtained from these experiments were then screened for their ability to decarboxylate certain amino acids to produce vasoactive amines, and they were subsequently identified by 16S rRNA gene sequence analysis.

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Anaerobic incubation of cecal contents. Equine cecal contents were obtained from adult horses which had been euthanized for reasons other than gastrointestinal disease, and put into sterile containers flushed with nitrogen gas, with care being taken to preserve anaerobic conditions. The contents were chilled to approximately 4°C for transport to the laboratory. All of the incubation experiments were carried out under oxygen-free conditions, at 37°C (Mk 3 anaerobic work station [Don Whitley Scientific Ltd., Basingstoke, Hampshire, United Kingdom] gassed with 80% nitrogen, 10% hydrogen, and 10% carbon dioxide).

The cecal contents were strained to remove the coarser fibrous material (over 0.5 cm in length) and then divided into 100-ml aliquots. In the first set of investigations, either corn starch (1 g/100 ml) or inulin (1 g/100 ml) was added to separate aliquots, with a third aliquot incubated without added carbohydrate to act as a control. Preliminary studies were undertaken to examine the effects of different carbohydrate concentrations on cecal fermentation, and 1 g/100 ml was found to be sufficient to give maximal effects. Cecal contents were incubated for 24 h at 37°C while being agitated (orbital shaker; Jencons-PLS Ltd., Leighton Buzzard, Bedfordshire, United Kingdom), as previously described (5), and during this time the pH of the fluid in each aliquot was measured at 2-h intervals to establish that carbohydrate fermentation was occurring.

Samples were taken from the cecal contents at 0, 6, 12, 18, and 24 h for enumeration of those streptococci, lactobacilli, and gram-negative anaerobes which would grow on standard selective media. For growth of streptococci, a Columbia blood agar with a streptococcus-selective supplement containing colistin sulfate and oxolinic acid (final concentrations, 5 and 2.5 mg/500 ml medium, respectively; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) was used. For the growth of lactobacilli, LAMVAB agar was used, made in accordance with the methods of Hartemink et al. (19). This was a medium with low pH and containing vancomycin to inhibit the growth of other gram-positive bacteria such as enterococci. In initial studies, three other selective media were also tested: de Man, Rogosa, and Sharpe agar (MRS) (12); Rogosa agar (38); and tomato juice agar (10). For growth of gram-negative anaerobes, Wilkins-Chalgren anaerobe agar (44) was used with a gram-negative anaerobe-selective supplement containing vancomycin and nalidixic acid (final concentrations, 10 and 2.5 µg/ml of medium, respectively; Oxoid Ltd.).

Bacterial numbers were determined by a dilution method, adapted from a technique originally used by Miles and Misra (30). Serial dilutions of cecal contents in sterile reduced physiological saline (0.5 g of cysteine hydrochloride/liter, pH 6.7) from $1:10^3$ to $1:10^{12}$ (vol/vol) were transferred to each of the selective media in triplicate. Following 2 to 3 days of incubation under anaerobic conditions at 37°C, the number of CFU were counted where they numbered between 3 and 30 for a particular dilution. The mean of the triplicate determinations was then used to calculate the number of CFU per ml of cecal contents. Cecal contents from 10 horses were used in these investigations, and data were expressed as the geometric means \pm standard errors of the means (SEM) and plotted against time.

In a second set of studies, virginiamycin (10 μ g/ml) or calcium hydrogen phosphate (CaHPO_4 ; 0.3 g/100 ml) was added to aliquots of cecal contents to which either starch or inulin had been added (both 1 g/100 ml). Other aliquots, to which 1 g of carbohydrate/100 ml or no carbohydrate had been added, acted as positive and negative controls, respectively. The cecal contents were incubated for 24 h, and samples were taken for determination of bacterial numbers as before. Cecal contents taken from four different animals were used in these experiments, and changes in bacterial numbers over the 24-h period were expressed as the geometric mean \pm SEM.

Determination of decarboxylase activity in streptococci and lactobacilli. Colonies of streptococci and lactobacilli, taken from cecal contents from four different horses and incubated for 24 h with either starch or inulin (both at 1 g/100 ml), were grown on streptococcus-selective, LAMVAB or MRS agars. Approximately 100 colonies, representing a range of morphologies, were subcultured and stored in Microbank tubes (Prolab Diagnostics, South Wirral, Cheshire, United Kingdom) at 80°C until required.

Decarboxylase activity in bacterial isolates was assessed using the methods of Bover-Cid and Holzapfel (8). Prior to the screening assays, isolates were grown in an activation medium consisting of MRS broth containing 5 mg of pyridoxal-5-phosphate/100 ml and 100 mg of each of the five amino acids (l-isomers of phenylalanine, tryptophan, tyrosine, isoleucine, and valine)/100 ml. The isolates were incubated anaerobically at 37°C and subcultured 5 to 10 times at 24-h intervals to maximize decarboxylase enzyme activity. Isolates were then transferred onto 24-well plates containing decarboxylase screening agars (8), each containing only one of the above-mentioned amino acids, plus bromocresol purple pH indicator. Following 72 h of incubation, a color change in the medium from brown to purple, representing a pH rise, was considered a positive result. A high-pressure liquid chromatography (HPLC) method (previously described [5]) was used to confirm the presence of the corresponding amines (phenylethylamine, tryptamine, tyramine, 3-methyl butylamine, and isobutylamine) in several representative samples of activation broth in which decarboxylase-positive bacteria had been subcultured.

Identification of decarboxylase positive isolates. Those bacterial isolates producing a positive color change in the presence of one or more of the amino acids in the screening media were further identified. After ensuring that the streptococci and lactobacilli were pure strains of catalase-negative gram-positive cocci or rods, respectively, the isolates were compared according to their profile of carbohydrate metabolism. This was achieved using the Rapid Strep and the API50 CH kits (both from BioMerieux UK Ltd.,

Basingstoke, Hampshire, United Kingdom), carried out according to the manufacturer's instructions. These tests were not used to obtain a definitive identification of the isolates, but where several isolates were found to have the same profile of carbohydrate metabolism, it was assumed that they were the same species, and only one of the isolates was used for further typing.

The representative samples of bacterial isolates identified as being positive for decarboxylase activity were then identified using sequence data from the first 500 bp of the 16S rRNA gene, performed by NCIMB Ltd., Aberdeen, United Kingdom (41). The sequence obtained for each isolate was identified by database comparison (BLAST search) using the GenBank nucleotide database (3).

Statistical analyses. All statistical comparisons were performed using GraphPad Prism, version 3.00, for Windows (GraphPad Software, San Diego, Calif.).

The effects of the two carbohydrate sources on bacterial numbers were compared to the controls by means of two-way (repeated-measures) analysis of variance followed by Bonferroni's post hoc test. The effects of the inhibitors, virginiamycin and calcium phosphate, were assessed at the 24-h time point, comparing the results obtained in the presence of the inhibitors to the positive and negative controls using one-way analysis of variance with Fisher's multiple comparison. In all cases, significance was accepted at $P < 0.05$.

Materials. All selective growth media and supplements were obtained from Oxoid Ltd., apart from the ingredients for the LAMVAB agar and decarboxylase screening medium, which were obtained from Merck Ltd., Lutterworth, Leicestershire, United Kingdom. Virginiamycin, CaHPO_4 and amino acids were obtained from the Sigma Chemical Company, Poole, Dorset, United Kingdom.

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Effect of carbohydrate on bacterial numbers. LAMVAB medium was chosen for the isolation and enumeration of lactobacilli, as the growth of some acid-tolerant streptococci from the equine cecum was not prevented on the other media tested. The numbers of streptococci, lactobacilli, and gram-negative anaerobic bacteria did not vary significantly in the control aliquots over the duration of the 24-h experimental period. The addition of starch or inulin resulted in a significant increase in the numbers of streptococci and lactobacilli over the 24-h period of incubation, by factors of 2 to 3.5 log units (Fig. 1a and b) and caused a significant drop in pH (1.5 ± 0.2 and 1.7 ± 0.3 pH units in the presence of starch and inulin, respectively). The numbers of gram-negative anaerobic bacteria were not significantly affected by carbohydrate overload (Fig. 1c).

Effect of inhibitors on bacterial numbers. The effects of virginiamycin (10 $\mu\text{g/ml}$) and calcium hydrogen phosphate (0.3 g/100 ml) are shown in Fig. 2a and b, respectively.

Virginiamycin (10 µg/ml) significantly inhibited the increase in numbers of streptococci induced by starch and inulin by 98.4 and 97.8%, respectively, and inhibited the increase in lactobacilli by 96.6 and 98.4%, respectively (Fig. 2). Calcium hydrogen phosphate (0.3 g/100 ml) had no significant effect on bacterial numbers, although there appeared to be a slight inhibitory trend.

Identification of amino acid decarboxylase-producing strains of streptococci and lactobacilli. Approximately 100 different isolates of streptococci and lactobacilli from four separate experiments were screened for the ability to decarboxylate one or more of the five amino acids incorporated into the screening agar medium. A total of 26 isolates produced a color change indicative of decarboxylase activity. HPLC analysis of five samples of activation broth in which these isolates had been subcultured showed increased production of the relevant amine(s) in the region of 1.5- to 10-fold.

A further screening of the decarboxylase-positive isolates, using the Rapid Strep or API50CH kits, demonstrated that the 26 isolates could be placed in 10 different groups on the basis of substrate fermentation. 16S rRNA gene sequencing and subsequent species identification was performed on representative isolates of these 10 fermentation groups.

The species of streptococci and lactobacilli identified by this method (including BLAST search score) are shown in Table 1, combined with the results of the decarboxylase screening assay. The species identified included *Streptococcus bovis* and five different *Lactobacillus* spp., most strains of which were from the species *Lactobacillus mucosae*. One isolate contained a sequence most closely matched with that of *Lactobacillus salivarius*, although the similarity of the sequence compared with that previously published was only 89.61%.

Subjectively, the most intense color changes were produced by the decarboxylation of tyrosine by isolates belonging to the species *L. mucosae* and *Lactobacillus reuteri* plus the isolates with similarities to *L. salivarius*, although there was no distinct species-related pattern to the amino acids which were metabolized.

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It has been shown that a wide range of potentially vasoactive amines occur at relatively high concentrations within the cecal contents of horses and that the concentrations of phenylethylamine and isoamylamine in particular were increased under conditions providing excess carbohydrate in vitro (5). We therefore postulated that the carbohydrate-fermenting bacteria, *Streptococcus* spp. and *Lactobacillus* spp., might, at least in part, be responsible for their production. The present study confirmed that numbers of streptococci and lactobacilli markedly increased in this model system following the addition of fermentable carbohydrate. Certain strains of these bacteria were shown to be

capable of producing one or more amines which may be of pathological importance due to their vasoactive properties.

Excessive amounts of fermentable carbohydrate (corn starch), administered orally to experimental horses, has been shown to cause lactic acid production within the cecum, associated with an overgrowth of gram-positive bacteria, particularly *Streptococcus* spp. and *Lactobacillus* spp. (15, 16). These changes often lead to the development of acute laminitis some 36 to 48 h later (14). Similarly, inulin (a naturally occurring form of fructan carbohydrate of a type similar to that found in grass) has also been used to initiate this condition (C. Pollitt, personal communication), and it has been suggested recently that fructans may be responsible for initiating pasture-induced laminitis (Longland et al., Proc. 16th Equine Nutr. Physiol. Soc.).

The in vitro model used in the present study was similar to those in other studies examining rumen contents from cattle and sheep, in which an excess of fermentable carbohydrate will also cause toxicity and acidosis (2, 13). Indeed, the bacterial flora of the equine cecum shows many similarities with that of the bovine rumen (26, 27). Fructans have also been shown to cause fermentation in the ceca of monogastric animals such as the rat (36), due to the fact that such oligosaccharides are not digestible in the mammalian small intestine.

The conditions under which the cecal contents were incubated did not lead to significant changes in pH or in the numbers of the bacterial species of interest in the control aliquots over the duration of the experimental period. The addition of starch or fructans to the cecal contents resulted in a marked fall in pH over the time course of the experiments (5), the magnitude of which is consistent with the cecal pH changes due to lactic acid production demonstrated in vivo when horses have been administered large amounts of starch (15, 31).

The fermentation of starch or fructans added to cecal contents was associated with marked increases (2 to 3.5 log units) in numbers of streptococci and lactobacilli. The increase in numbers of streptococci and lactobacilli in the present study is consistent with the results of those in vivo experiments (16). In those studies by Garner et al., however, the numbers of gram-negative anaerobic bacteria fell following the administration of carbohydrate, thought to be due to lactic acid production causing their death and subsequent release of endotoxin. The numbers of gram-negative anaerobic bacteria counted in cecal contents from the present study did not decline significantly, although it may be that more-prolonged exposure to conditions of low pH might overcome their apparent pH tolerance and result in their decline. The total number of gram-negative anaerobic bacteria isolated in the present study was in line with a previous report enumerating equine cecal bacteria (25).

The rate of increase of gram-positive bacterial numbers correlated with the fall in pH and the increase in concentrations of phenylethylamine and isoamylamine previously described (5), and it is known that gram-positive bacteria, such as streptococci and lactobacilli, have the ability to produce amines from amino acids (8, 18). It should be noted however, that concentrations of these and other amine compounds were already high in cecal contents from normal horses relative to their potential effects on equine

digital blood vessels (Berhane et al., Abstr., Br. J. Pharmacol. **135**:291P; Bailey et al., J. Vet. Int. Med. **15**:374, 2001). Therefore, it should not be concluded that these particular gram-positive bacteria were the sole source of production of these compounds. Indeed, many other groups of bacteria, including groups of gram-negative bacteria such as the enterobacteria, contain the decarboxylase enzymes necessary to produce amines (17). Nevertheless, the marked increase in the numbers of streptococci and lactobacilli associated with carbohydrate fermentation suggests that these bacteria may be of great significance in the production of vasoactive amines under these conditions.

The streptogramin antibiotic, virginiamycin, inhibited the increases in numbers of streptococci and lactobacilli associated with carbohydrate excess in the present study, as it did the production of phenylethylamine and isoamylamine (5). It did not appear to decrease numbers of streptococci or lactobacilli to levels below the numbers observed in control aliquots, however. Virginiamycin was included at a concentration of 10 µg/ml, based upon the concentration shown to have effects on rumen fermentation in vitro (29) and its MIC for *S. bovis* and *Lactobacillus* spp. cultured from the rumen (34). Orally administered virginiamycin moderates rumen fermentation in vivo (11), and in the horse, this antibiotic has been marketed in Australia as Founderguard, an oral formulation for the prevention of laminitis. This product has been shown to suppress lactic acid production in the hind gut of horses caused by grain overload (40).

As a comparison to virginiamycin, the effects of calcium hydrogen phosphate (CaHPO_4) were also investigated. Dietary calcium in the form of CaHPO_4 has been shown to ameliorate inulin-induced cecal fermentation in the rat, partly by control of luminal pH (36). Insoluble CaP_i did not otherwise affect microbial activity. In our model, CaHPO_4 significantly limited the fall in pH associated with fermentation of both starch and inulin by equine cecal contents (5). There was no significant effect on bacterial numbers in the present study, however, although a trend was seen. Taken together, these results confirm that the significant effects of virginiamycin on amine production were due to specific antimicrobial properties, rather than being indirect through the moderation of pH.

The selective culture media for the growth of the three groups of bacteria examined in this study appeared to show good sensitivity, with LAMVAB agar providing better selective growth of cecal lactobacilli than MRS and Rogosa agars. Twenty-six strains of lactobacilli and streptococci from four different animals gave positive reactions for decarboxylase activity in the presence of one or more of the amino acids when grown on the decarboxylase screening agar. Several different amino acids were examined in this procedure, corresponding to the production of different vasoactive amines, since there is substrate selectivity among the different amino acid decarboxylase enzymes (7). Interestingly, once the bacterial species had been identified by 16S rRNA gene sequence analysis, it became clear that there was no species-specific pattern to the combination of amino acids converted. This finding has previously been reported by Bover-Cid et al. (9), who also noted that decarboxylase activity in lactobacilli tended to be dependent on strain rather than species.

Of the four isolates of *Streptococcus* spp. identified as having the ability to decarboxylate one or more of the amino acids tested, all were found by sequence analysis to be of the species *S. bovis* (43). *S. bovis* has been implicated in the pathogenesis of acute laminitis

in the horse, being one of the principal species found to overgrow in the ceca of horses given corn starch to experimentally induce this condition (1). In addition, a recent paper by Mungall et al. (33), in which supernatants of bacterial broth in which *S. bovis* had been grown were added to hoof lamellar explants, described that laminar separation occurred as a result of matrix metalloproteinase activation. The present study therefore provides another possible means by which *S. bovis* may play a role in causing laminitis.

The majority of strains of lactobacilli able to decarboxylate one or more of the added amino acids in the present study belonged to the species *L. mucosae*. This is a relatively new species, initially isolated from the pig intestine (39), and not previously described in the horse hindgut. It is, however, closely related to other species such as *L. reuteri* and *Lactobacillus fermentum* (39), which were also found to have decarboxylase activity in the present study. These species, together with *L. salivarius* and *Lactobacillus delbruekii*, have all been shown to be responsible for the production of amines in various fermented foodstuffs (8, 42). The strong positive color change for the decarboxylation of tyrosine by a number of strains of lactobacilli may reflect the extent to which these bacteria produce tyramine (32). However, this method was not considered to be of quantitative value, since the concentration of tyramine in some samples of activation broth measured by HPLC appeared to be no greater than that of other amines.

In summary, this study has demonstrated that the addition of starch or fructan carbohydrates to equine cecal contents was associated with a marked increase in the numbers of streptococci and lactobacilli over 24 h, some of which were capable of decarboxylating certain amino acids to produce amine compounds. Their growth was markedly inhibited by virginiamycin, but not by calcium hydrogen phosphate. The vasoactive properties of some of these amines suggest that their absorption from the cecum into the circulation of the horse, following fermentation of excess carbohydrate, could trigger changes in blood flow to the digit and therefore could be involved in the pathogenesis of acute laminitis. Additional studies are warranted to investigate further the role of vasoactive amines in this disease and the conditions favoring their production in the equine hindgut.

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FIGURES AND TABLES

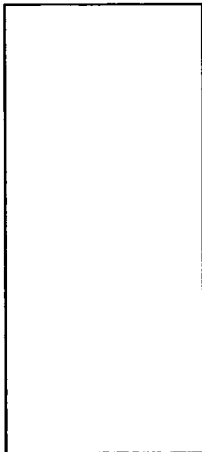


FIG. 1.

Effects of carbohydrate overload on numbers of streptococci (a), lactobacilli (b), and gram-negative anaerobes (c) in equine cecal contents incubated anaerobically *in vitro*. Cecal contents were divided into aliquots and incubated for 24 h with the inclusion
([more ...](#))

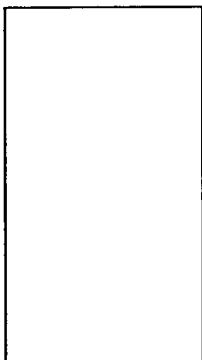


FIG. 2.

Effects of virginiamycin and calcium hydrogen phosphate on numbers of *Streptococcus* (a) and *Lactobacillus* (b) organisms in equine cecal contents induced by incubation with starch or inulin. Aliquots of cecal contents were incubated with carbohydrate in (more ...)

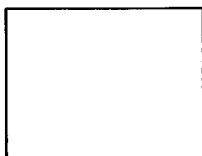


TABLE 1.

Conversion of amino acids to amines by strains isolated from equine cecal contents incubated with starch or inulin^a